

Dipyridamole inhibits human peritoneal mesothelial cell proliferation in vitro and attenuates rat peritoneal fibrosis in vivo

KUAN-YU HUNG, REN-SHI SHYU, CHENG-CHUNG FANG, CHIEN-CHEN TSAI, PO-HUANG LEE, TUN-JUN TSAI, and BOR-SHEN HSIEH

Departments of Internal Medicine, Emergency Medicine, Surgery, and Pathology, National Taiwan University Hospital, Taipei, Taiwan, Republic of China

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Background. Peritoneal fibrosis (PF) is one of the most serious complications after long-term continuous ambulatory peritoneal dialysis (CAPD). Proliferation of human peritoneal mesothelial cells (HPMC) and matrix over-production are regarded as the main processes predisposing to PF. Dipyridamole (DP) has been reported to have potential as an antiproliferative and antifibrotic agent. We thus investigated the effect of DP in inhibiting proliferation and collagen synthesis of HPMC. A rat model of peritonitis-induced PF was also established to demonstrate the in vivo preventive effect of DP.

Methods. HPMC was cultured from human omentum by an enzyme digestion method. Cell proliferation was measured by the methyltetrazolium assay. Intracellular cAMP was measured using an enzyme immunoassay (EIA) kit. Total collagen synthesis was measured by ³H-proline incorporation assay. Expression of collagen $\alpha 1$ (I) and collagen $\alpha 1$ (III) mRNAs was determined by Northern blotting. The rat model of peritonitis-induced PF was developed by adding dextran microbeads (Cytodex, 8 mg/1 mL volume) to a standardized suspension (3×10^9) of *Staphylococcus aureus*. DP was administered via intravenous infusion (4 mg in 1 h) daily for seven days. Macroscopic grading of intraperitoneal adhesions and histological analyses of peritoneal thickness and collagen expression were performed.

Results. Addition of DP to HPMC cultures suppressed serum-stimulated cell proliferation and collagen synthesis. The antimitogenic and antifibrotic effects of DP appear to be predominantly mediated through the cAMP pathway, as DP increased intracellular cAMP in a dose-dependent manner. The macroscopic grade of intraperitoneal adhesion and peritoneal thickness were both significantly increased in animals treated with Cytodex plus *S. aureus*; on the other hand, DP attenuated these fibrotic changes with statistical significance ($P < 0.01$). Analysis of gene expression of collagen $\alpha 1$ (I) and $\alpha 1$ (III) in

the peritoneal tissue of experimental animals yielded similar results.

Conclusions. This study suggests that dipyridamole may have therapeutic potential in treating peritoneal fibrosis.

Continuous ambulatory peritoneal dialysis (CAPD) has gained increasing popularity in the treatment of uremia, and peritoneal fibrosis (PF) is one of the most serious complications after long-term CAPD [1]. It had been postulated that proliferation of human peritoneal mesothelial cells (HPMC) accompanied by matrix expansion plays a fundamental role in the pathogenic process of PF [2]. Pharmacological interventions that can inhibit the proliferation of HPMC and/or matrix formation thus may be beneficial for the prevention or retardation of the progression of PF.

Dipyridamole (DP; 2,6-bis[diethanolamino]-4,8-dipyridinopyrimido-[5,4-d]-ymidine) is an antiplatelet agent with an undefined mechanism of action [3]. In addition to its antiplatelet activity, DP has been shown to exert antiproliferative effects on rat vascular smooth muscle cells [4] and human mesangial cells [5]. We have previously shown that DP exerts an antifibrogenic effect on rat mesangial cells by inhibiting cell proliferation and reduce cell expression of type I collagen mRNA [6]. However, the inhibitory effect of DP on HPMC proliferation has never been reported. Accordingly, the first aim of the present study was to investigate in vitro the inhibitory effect of DP on HPMC proliferation and collagen accumulation.

The importance of bacterial peritonitis as a leading etiological agent of PF in long-term CAPD patients is well established [7]. Several studies have demonstrated that *Staphylococcus aureus* is one of the most common organisms causing CAPD peritonitis. An experimental infection model of *S. aureus* peritonitis was attempted

Key words: mesothelial cell, collagen, animal model, CAPD, mitogenesis, cAMP, uremia, extracellular matrix.

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by Calame et al [8]; however, their infection model was less severe than ours and no study has focused specifically on the pathogenesis of PF. Another rat model of PF induced by *Escherichia coli* has also been postulated [9]. Nevertheless, such infection is rare in the CAPD population and the induction of PF was not successful. Therefore, we sought to establish a model that more closely mimicked peritonitis-related PF in CAPD patients. The second aim of our work was to establish a rat model of PF developed secondary to *S. aureus* peritonitis. The third aim was to demonstrate the in vivo preventive effect of DP on this experimental PF rat model.

METHODS

Materials

Fetal calf serum (FCS) was obtained from Biochrome KG (Berlin, Germany). Culture flasks and plates were purchased from Corning (Corning, NY, USA) and pre-coated with 1.6 µg/cm² of Vitrogen 100® (Celtrix Lab, Palo Alto, CA, USA) before cell loading. Trypsin-ethylenediaminetetraacetic acid (EDTA), RPMI-1640 medium, glutamine, and trypan blue were obtained from Gibco (Grand Island, NY, USA). Bovine serum albumin (BSA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 3-isobutyl-1-methylxanthine (IBMX) and other tissue culture reagents were purchased from Sigma (St. Louis, MO, USA). ³H-proline was purchased from DuPont NEN (Boston, MA, USA). The enzyme immunoassay (EIA) kits for cyclic adenosine monophosphate (cAMP) were obtained from Cayman Chemical (Ann Arbor, MI, USA). Human collagen α1 (I) and collagen α1 (III) cDNAs were purchased from American Type Culture Collection (Rockville, MD, USA). Agents used to isolate the total RNA and Northern blot analysis were obtained from Boehringer Mannheim (Mannheim, Germany) unless otherwise specified. DP was generously provided by Boehringer Ingelheim (Ingelheim, Germany). All other chemicals used were of analytical grade.

Human peritoneal mesothelial cell culture

Specimens of human omentum were obtained from abdominal surgical procedures for elective gastric cancer resection and the omentum was essentially normal. The HPMC culture was carried out as we previously reported [10, 11]. Briefly, the surgically removed human omentum was washed three times with PBS and then digested with trypsin EDTA (0.125%) for 15 minutes. After centrifugation, the cell pellet was washed with culture medium and then seeded into a vitrogen-coated (1 mg/mL) flask. The medium was changed on the third day. RPMI-1640 medium containing 20% FCS, penicillin (100 U/mL), streptomycin (100 µg/mL), and insulin (30 µg/mL) was used. After two to four days, the cells became confluent and were subcultured with medium containing 10% FCS.

HPMC were identified by the presence of vimentin and cytokeratin without desmin or factor VIII-related antigen using the immunofluorescence method. All experiments were performed in cells from passages 1 to 3.

Cell proliferation assay

A modified MTT assay was used to evaluate PMC proliferation as previously reported [10]. The amount of MTT uptake (absorbance at 570 nm) by HPMC was found to vary linearly with cell numbers ranging from 4000 cells/well to 12.8 × 10⁴ cells/well in 96-well plates [10]. We loaded 5000 cells/well for each MTT assay. Cells growing in the log phase were trypsinized and plated down in 96-well plates with RPMI-1640 medium containing 10% FCS. Various concentrations of treating agents were added after overnight plating. The medium and drug were changed every three days. After additional incubations of 24 to 120 hours, 20 µL MTT solution (5 mg/mL in PBS) was added to the culture medium. Cells were incubated further at 37°C for four hours, after which the medium was replaced by 100 µL ethanol. Absorbance at the reference wavelength of 630 nm and test wavelength of 570 nm was measured by an enzyme-linked immunosorbent assay (ELISA) reader. All samples were tested in pentaplicate. The inhibition of HPMC growth was calculated as follows [10]:

$$\text{Percentage of inhibition} = 1 - \frac{\text{Absorbance of (test well - initial plating)}}{\text{Absorbance of (control - initial plating)}} \times 100\%$$

Intracellular cyclic-AMP assay

We loaded 3 × 10⁵ HPMC cells/well into a six-well plate with RPMI-1640 medium containing 10% FCS. After cells became subconfluent, they were washed twice with warm RPMI-1640 medium and then mixed with various concentrations of agents. After five minutes of incubation, the supernatants were discarded and ice-cold 95% methanol was added to each well. After 30 minutes of incubation at 4°C, the supernatants evaporated and cAMP was measured using EIA kits. The cells in wells were lysed by 0.1 N NaOH and protein content was measured by bicinchoninic acid assay, using BSA as a standard [12]. All experiments were performed four times.

Assay of total collagen synthesis

A ³H-proline incorporation assay was used to measure total collagen production, as we previously described [11]. Briefly, HPMC were seeded in 96-well plates at a density of 2.5 × 10⁴ cells/well in 200 µL RPMI-medium supplemented with 10% FCS. After 48 hours, cells became confluent. Then the medium was replaced with ascorbic acid (50 µg/mL) in the presence of various concentrations of DP. After incubation for another 48 hours,

cells were labeled with 0.5 μ Ci of ^3H -proline (100 Ci/mmol) and 50 $\mu\text{g/mL}$ β -aminopropionitrile during the final 24 hours of incubation. ^3H -proline incorporation into pepsin-resistant, salt-precipitated collagen was measured with a liquid scintillation counter. The cell numbers were determined simultaneously by MTT assay in identically treated microplates. The results were expressed as disintegrations per minute (dpm) of ^3H -proline per 10^4 cells and were compared to the control values. All experiments were performed in triplicate.

Northern blot analysis

To determine the effect of DP on collagen gene expression, HPMC were grown in RPMI supplemented with 10% FCS until subconfluency. HPMC were then treated with fresh medium in the absence or presence of various concentrations of DP. At indicated time intervals, cells were harvested for isolation of total RNA, as previously described [11]. The concentration of each sample was determined using spectrophotometry with the absorbance at 260 nm (A_{260}). The purity of each sample was determined based on the ratio of A_{260} to A_{280} . Ten micrograms of RNA were electrophoresed on a 1% agarose gel containing 1 mol/L formaldehyde in MOPS buffer (0.2 mol/L morpholinopropanesulfonic acid, 0.05 mol/L Na acetate, 0.01 mol/L EDTA). Equivalency of sample loading and lack of degradation were verified by ethidium bromide staining of the 28 S and 18 S rRNA bands. The RNA then was transferred to nylon membranes by overnight capillary action and followed by fixation in a UV cross-linker.

Because HPMCs mainly express type I and III collagens [13], both of these were used as probes for Northern blotting. A 1.5-kb EcoRI fragment of collagen $\alpha 1$ (I) and a 0.7-kb HindIII/EcoRI fragment of collagen $\alpha 1$ (III) were subcloned, respectively, into pBSII/SK (Stratagene, La Jolla, CA, USA) and used as templates for in vitro transcription of antisense digoxigenin-conjugated riboprobes (Boehringer Mannheim) according to the manufacturer's instructions. The blots were developed using CSPD (Boehringer Mannheim) as the substrate for alkaline phosphatase, as described by the supplier. The signal intensity recorded on x-ray film was then quantified with computerized densitometry and normalized against the signal of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messages.

Animal model

Animals. Male Wistar rats (weighing 180 to 200 g; Simonson Lab., Gilroy, CA, USA) were maintained on a 12-hour light/dark cycle and provided with food and water ad libitum. All work was carried out in accordance with American Association for Accreditation for Laboratory Animal Care (AAALAC) regulations.

Preparation and induction of peritonitis. The *S. aureus*

strain ATCC 25923 (American Type and Culture Collection, MD, USA) was used throughout the study. Before each experiment, a fresh six to nine hour culture in a brain-heart infusion broth (BHI, Oxoid, Basingstoke, UK) was made in an agitated water bath at 37°C. Serial dilutions were prepared and plated to determine the actual inoculum sizes used. To enhance local bacterial infection in the rat peritoneal cavity, dextran beads (Cytodex; Sigma Chemicals, St. Louis, MO, USA) were added to the bacterial suspension for inoculation as described by Ford et al [14]. Beads were first prepared as a stock solution by suspending 1 g in 50 mL of PBS, and were autoclaved before use. Aliquots of the Cytodex suspension were added to the broth with or without bacteria (3×10^9 CFU), and then additional broth was added for a final volume of 1 mL for the inoculation. Rats were divided into groups, tagged, and given an intraperitoneal (IP) injection of the prepared suspension with a 1 mL tuberculin syringe.

Pilot study to establish the experimental protocol. In a pilot experiment, 30 rats were used to establish the experimental model of peritonitis and induction of PF. To examine the toxicity of Cytodex, six rats that had received IP injections of various volumes and concentrations of Cytodex suspension were observed for 72 hours. Another six rats received continuous intravenous infusion (IVF) of DP (4 mg in 1 hour) via a jugular vein catheter, which did not produce side effects. During the observation period, all rats were active and no deaths were recorded. After being anesthetized with ketamine [75 mg/kg body weight intramuscularly (IM)], 18 rats received IP injections of various combinations of bacteria and Cytodex suspension solution. After this procedure, these animals were randomly allocated into two experimental groups, and were either left without further treatment or treated daily with IVF of DP through implanted jugular vein catheters. Through these pilot experiments, we expected to successfully induce a peritonitis-related PF. The induced peritonitis in rats was to be serious enough to cause the subsequent development of PF but not death. Based on the results of the pilot experiments, the experimental design for the in vivo animal study was adapted as described in the next section.

Experimental design. In each experiment, rats were divided into four groups of 10 rats per group. Group 1 received IP injections of bacteria (3×10^9 CFU) only, group 2 received Cytodex (8 mg/1 mL volume) only, group 3 received bacteria mixed with Cytodex (3×10^9 CFU bacteria plus 8 mg Cytodex in a total volume of 1 mL), and group 4 received the same manipulation as group 3 except with an additional daily IVF of DP (4 mg in 60 min) for seven days. The experiment was completed on day eight. Ten additional healthy rats that received IP injections of 1 mL 0.9% saline were used as the control group.

Morphological and histochemical analysis of the peritoneal cavity

Gross adhesion scoring system. After anesthesia, the rat abdomen was completely opened for an objective observer to assess the adhesions. For this purpose, a scale developed by Mullarniemi et al was applied with modifications [15]. A score of 0 indicated no adhesion, 1 indicated one to three separate adhesions, 2 indicated three or more distinctly separate adhesions, and 3 indicated diffuse, sheet-like adhesions. Based on the results of pilot experiments described earlier, we found that adhesions may develop between the liver and omentum, the omentum and intestinal wall, the intestine and abdominal wall, or between the intestine and stomach. Therefore, these fields were checked separately, making 12 the highest possible score. The total scores of each rat were recorded for statistical analysis.

Histological analysis. At the time of sacrifice, peritoneal tissue specimens were taken from the gut with the mesentery and from the liver for histological analysis. After being formalin fixed and paraffin embedded, tissue was cut into 5 μm thick sections and processed using a standard hematoxylin and eosin stain and Masson's Trichrome stain. The thickness of the connective tissue between the mesothelium and the liver surface or intestine wall was objectively evaluated, as previously reported [16, 17]. Each tissue section was measured at five random locations. Six tissue sections per rat (3 from the gut, 3 from the liver surface) were examined. The average thickness (μm) of each tissue section was recorded for statistical analysis between different groups.

Northern blot analysis of collagen content. Animals were sacrificed, and tissue samples of the omentum, adhesion strands, and the right upper quadrant of the abdominal wall were taken and then immediately frozen in an ethanol/dry ice bath and stored at -70°C . Total RNA was extracted from homogenized tissue using TRIzol reagent (Life Technology, Grand Island, NY, USA) according to the manufacturer's protocol. Total RNA (20 $\mu\text{g}/\text{lane}$) was electrophoresed on a 1% agarose/1 mol/L formaldehyde gel and then transferred to a nylon membrane. Hybridization, washing, and autoradiography were performed as described previously for Northern blot analysis in cultured HPMC.

Statistical analysis

Results are expressed as mean \pm SD, unless stated otherwise. Statistical analyses were carried out using SPSS/Windows (SPSS Inc.) and StatView 4.1 (Abacus Concept Inc., Berkeley, CA, USA) software on a personal computer. Statistical significance ($P < 0.05$) was evaluated by the Student t test or one-way ANOVA and modified t test with application of the Bonferroni correction.

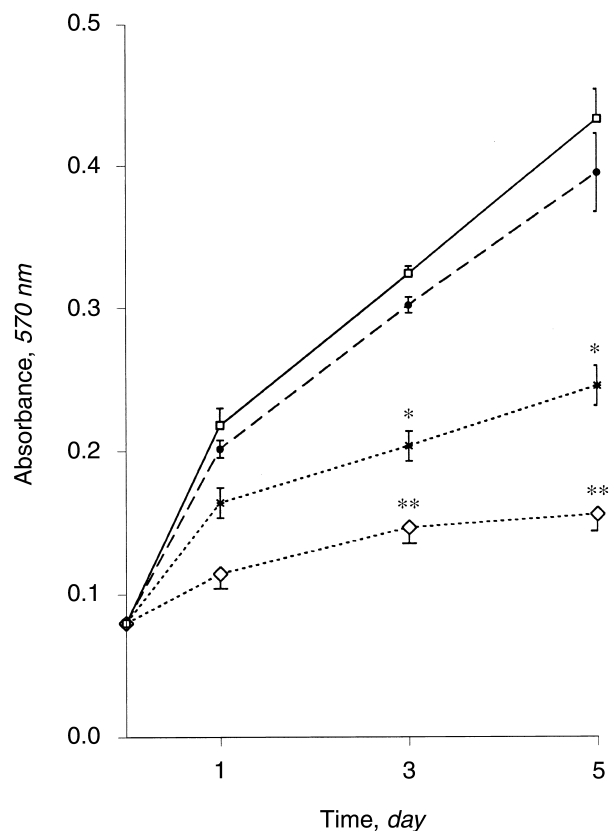


Fig. 1. Dipyridamole (DP) inhibits human peritoneal mesangial cell (HPMC) growth. Time-response curve: Varying concentrations of DP were added after overnight plating of HPMC and further incubated with 10% fetal calf serum (FCS) for one to five days. The experiment was repeated four times. Symbols are: (□) 10% FCS; (●) 10% FCS + 2 $\mu\text{g/mL}$ DP; (■) 10% FCS + 6 $\mu\text{g/mL}$ DP; (◇) 10% FCS + 17 $\mu\text{g/mL}$ DP; * $P < 0.05$ and ** $P < 0.01$, compared to 10% FCS.

RESULTS

In vitro studies

Dipyridamole inhibited serum-stimulated proliferation of HPMC. Growth curve experiments were performed to evaluate the effect of DP on serum-stimulated HPMC proliferation. In time response experiments (Fig. 1), DP at concentrations above 6 $\mu\text{g/mL}$ caused a dose-dependent inhibition of HPMC. The dose-response study of the effect of increasing concentrations of DP on HPMC proliferation is shown in Figure 2. When compared to the control group (10% FCS), growth inhibition was statistically significant ($P < 0.05$) starting at 6 $\mu\text{g/mL}$ of DP. The percentages of inhibition by DP at concentrations of 6 and 17 $\mu\text{g/mL}$ were 44% and 53%, respectively. We therefore used a higher concentration (17 $\mu\text{g/mL}$) of DP in the following experiments to study its antifibrotic effect on HPMC. To exclude the possibly toxic effect of such a concentration of DP on proliferative HPMC, a cell viability test was performed using the trypan blue exclusion method and by measuring the lactate dehy-

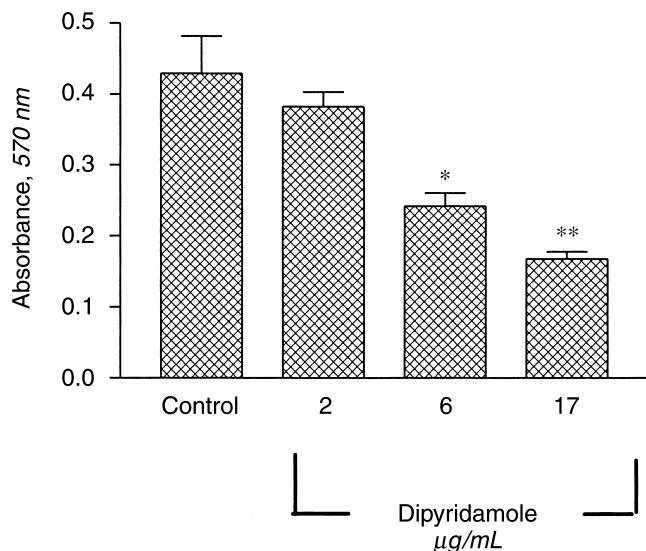


Fig. 2. Effect of DP on serum-stimulated HPMC proliferation. For the dose-response study, HPMC were incubated for 120 hours after addition of varying concentrations of DP. Data represent the mean \pm SD from three independent experiments performed in triplicate. * P < 0.05 and ** P < 0.01, compared to control.

drogenase (LDH) activity of the supernatant as we previously described [10, 11]. No differences were found in the numbers of dead cells in the supernatant and adherent HPMC fractions between control and DP-treated wells (data not shown).

Dipyridamole increased intracellular cAMP levels in HPMC

Dipyridamole is known to function as a phosphodiesterase inhibitor, thereby increasing intracellular cAMP levels [3, 5]. To evaluate whether DP increases intracellular cAMP levels in HPMC, we measured cAMP levels after incubation of HPMC with varying concentrations of DP (2, 6, and 17 µg/mL). In these experiments, IBMX (1 mmol), a known phosphodiesterase inhibitor, was used as a positive control (Fig. 3). cAMP levels were elevated in a dose-dependent manner by DP. The addition of DP at concentrations of 6 and 17 µg/mL resulted in a significant increase in the intracellular cAMP levels in HPMC. As expected, the addition of IBMX showed a significant increase in intracellular cAMP levels in HPMC.

Dipyridamole inhibited collagen synthesis of HPMC

As shown in Figure 4, total collagen synthesis by HPMC was inhibited by DP in a dose-dependent manner. Additionally, Northern blot analysis showed that DP decreased collagen α 1 (I) and collagen α 1 (III) mRNA levels (Fig. 5). The results of densitometry showed that, after 12 hours of incubation, DP decreased serum-stimulated collagen α 1 (I) mRNA expression by

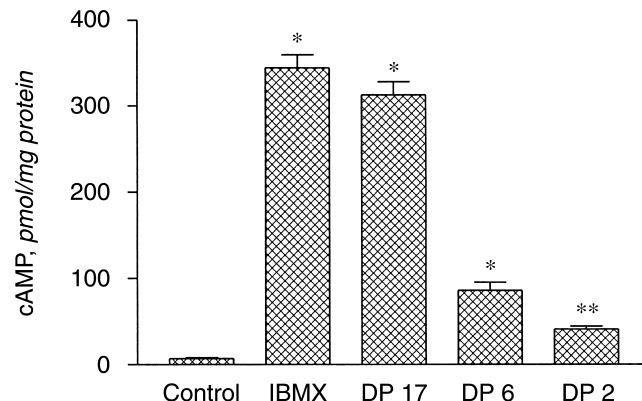


Fig. 3. Effect of DP (µg/mL) on intracellular cAMP levels (pmol/mg protein) as measured by a cAMP kit. Intracellular cAMP levels were elevated in a dose-dependent manner by DP. IBMX is 3-isobutyl-1-methylxanthine. Data represent the mean \pm SD from three independent experiments performed in triplicate. * P < 0.01 and ** P < 0.05, relative to control.

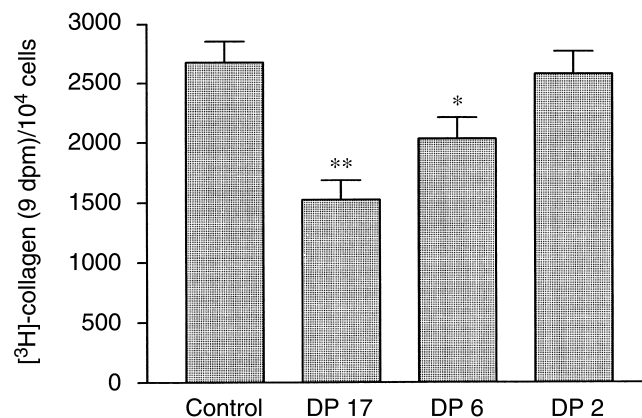


Fig. 4. Effect of DP (µg/mL) on total collagen production by HPMC. All values were normalized by cell number (10⁴ cells). * P < 0.05 and ** P < 0.01 relative to control. The experiment was repeated four times in triplicate.

26% (collagen/GAPDH ratio 1.15 vs. 0.85 for lane 1 vs. 3). After 24 hours of incubation, DP decreased (collagen/GAPDH ratio 0.6 and 1.27 for lanes 4 and 5, respectively) collagen α 1 (I) gene expression by 55% compared with that treated with serum alone. We have previously demonstrated a suppressive effect of collagen α 1 (I) mRNA expression by DBcAMP (1 mmol) in cultured HPMC [11]. In our current study, the expression of collagen α 1 (III) mRNA in HPMC was also suppressed after the addition of DBcAMP (1 mmol; data not shown).

In vivo studies

Gross adhesion scoring system. Figure 6 shows the results of the adhesion assessment in the different treatment groups. Group 1 (bacteria only) exhibited a mean score of 0.9 ± 0.18 , which was not significantly different

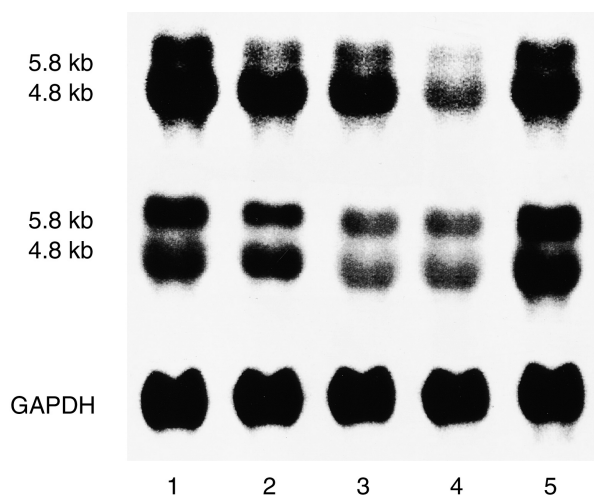


Fig. 5. Effects of DP on collagen $\alpha 1$ (I) and collagen $\alpha 1$ (III) mRNA levels. Lane 1, control at zero time; lanes 2–4, treatment of cells with DP (17 μ g/mL) for 6 hours, 12 hours, and 24 hours, respectively; lane 5, control at 24 hours.

from group 2 (Cytodex alone) with a mean score of 0.1. In contrast, the rats in group 3 (Cytodex and bacteria) had marked intraperitoneal adhesions with a mean adhesion score of 7.3 ± 0.42 . Treatment with DP (group 4) resulted in a significant reduction of the adhesion score to a mean of 1.5 ± 0.43 ($P < 0.01$, compared to group 3). In this group, three (30%) rats were free of adhesions. There was no significant difference in gross visual adhesion scores between the control (saline only, data not shown) and group 1 or 2 rats.

Histological analysis. Figure 7 shows photomicrographs of representative sections taken from animals after treatment. Upon histologic examination, the normal peritoneum consists of a single layer of mesothelial cells and their underlying submesothelial tissue. In the histology specimen of rats treated with saline alone (control), no fibrosis was observed (not shown). In comparison to the control group, the fibrosis was much more prominent in group 3 rats, which had advanced gross intraperitoneal adhesions. An increased connective tissue deposit, which appeared as a thickening between the fragmented mesothelial layer of the peritoneum and the liver surface, was noted (Fig. 7C). The images in Figure 7 E–H are representative of different treatment groups showing the arteries of the gut mesentery. In group 3 rats (Fig. 7G), the walls of the small arteries appeared much thicker than those in the other three treatment groups; however, no increases were observed in wall thickness in small arteries of the gut mesentery in the DP treated rats (Fig. 7H). Figure 8 shows a comparison of the mean connective tissue thickness between the different treatment groups. Animals in groups 1 and 2 had the lowest mean value of peritoneal connective tissue thickness, whereas group 3 rats had the highest value.

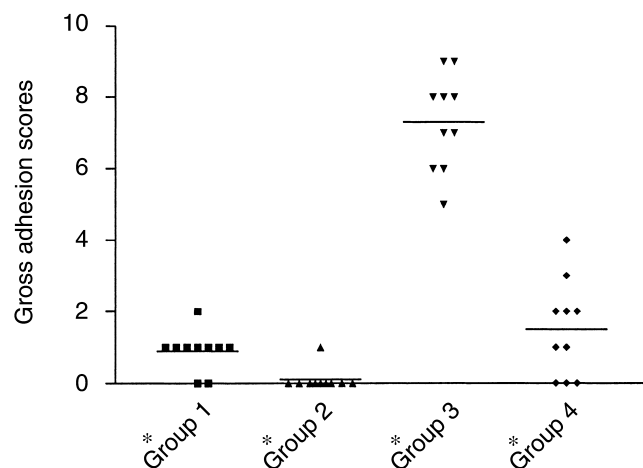


Fig. 6. Treatment of DP on adhesion formation in rats. The rat in group 3 (Cytodex mixed with bacteria) had the highest score (mean score 7.3 ± 0.42) of adhesion ranking, and the adhesion score was significantly reduced in rats treated with DP (group 4, mean score 1.5 ± 0.43). There was no significant difference between the group that received bacteria only (group 1) and the group that received Cytodex only (group 2); the means of the adhesion scores were 0.9 ± 0.18 and 0.1, respectively. * $P < 0.01$ compared to group 3.

Treatment with bacteria plus Cytodex (group 3) triggered a fourfold increase in connective tissue thickness compared to the bacteria-treated (group 1) or Cytodex-treated rats (group 2; $P < 0.01$). Rats treated with DP (group 4) had a significantly reduced connective tissue thickness (0.43 ± 0.04) compared with that of group 3 rats (0.96 ± 0.05 ; $P < 0.05$). These measurements revealed quantitative differences between groups, a result that is similar to that obtained by using the gross visual adhesion scoring system (Fig. 6).

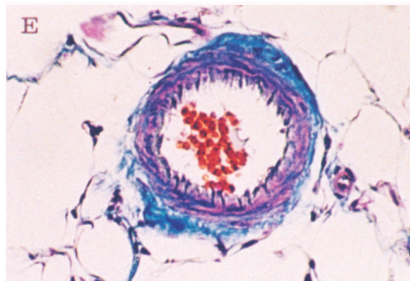
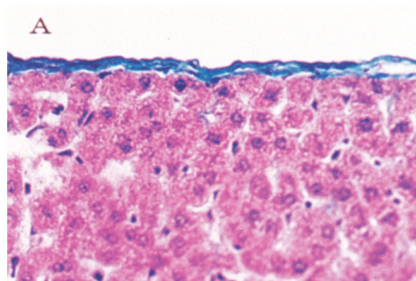
Northern blot analysis. Northern blot analysis was applied to detect collagen $\alpha 1$ (I)/ $\alpha 1$ (III) mRNA transcripts in peritoneal samples obtained from the four groups. The message expressed was significantly increased in samples from group 3 rats (bacteria + Cytodex treated) compared to that of group 1 (bacteria treated) or group 2 (Cytodex treated; Fig. 9). In contrast, the expression of both genes in peritoneal tissue was suppressed by DP (group 4).

DISCUSSION

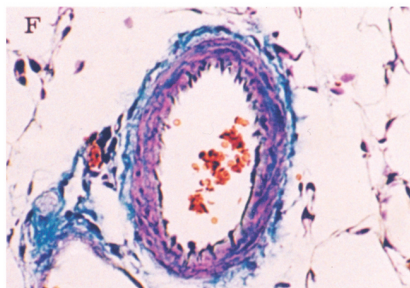
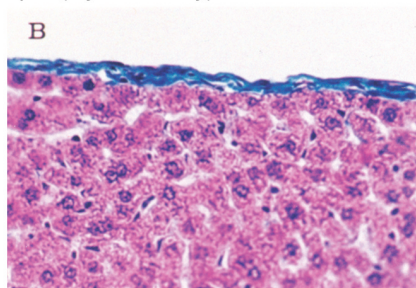
In vitro effects

This study demonstrated the inhibition of HPMC growth by DP (Figs. 2 and 3), an effect similar to what we previously reported in cultured rat mesangial cells [6]. DP also suppressed total collagen synthesis of HPMC in a dose-dependent manner (Fig. 5). By Northern blotting, the present study showed that DP attenuated the mRNA levels of collagen $\alpha 1$ (I) and collagen $\alpha 1$ (III) at 24 hours (Fig. 6). These in vitro effects of DP indicate that it has therapeutic potential for PF in vivo.

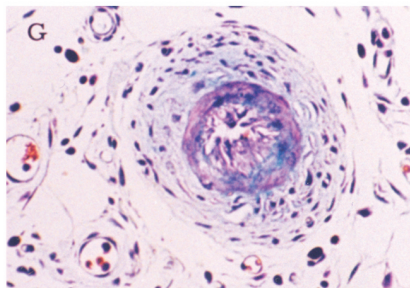
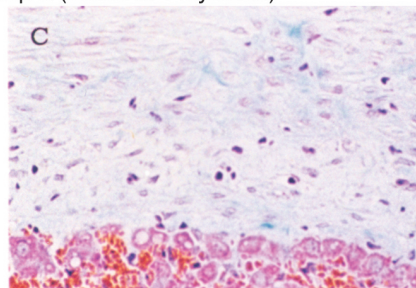
Group 1 (bacteria only)



Group 2 (Cytodex only)



Group 3 (bacteria + Cytodex)



Group 4 (bacteria + Cytodex + dipyridamole)

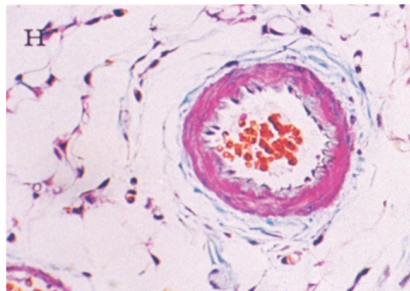
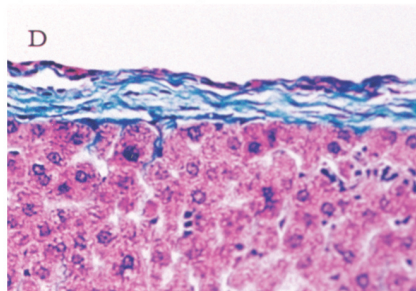


Fig. 7. Histological assessment of fibrotic reaction in peritoneal tissues. Representative sections of Trichrome-Masson-stained peritoneum displaying fibrotic thickening between the mesothelial layer and liver surface (A–D). Arteries of gut mesentery displaying different magnitudes of wall thickness attributable to fibrotic deposition in the four groups (E–H) (original magnification $\times 200$).

It has been suggested that DP acts as an antiplatelet agent by serving as a phosphodiesterase inhibitor that increases intracellular cAMP [3, 5]. Our current study demonstrates an increase of intracellular cAMP after DP treatment (Fig. 4), while our previous reports showed that raised intracellular cAMP could inhibit serum-stimulated HPMC proliferation and collagen synthesis [11], and also demonstrated that N-[2-((p-Bromocinnamyl) amino)ethyl]-5-isoquinolinesulfonamide (H-89), a protein kinase A (PKA) inhibitor, could reverse the antimetogenic and antifibrogenic effects of a phosphodiesterase

inhibitor (pentoxifylline) on HPMC [18]. These data combined indicate that the effect of DP may be through the cAMP pathway. The influence of cAMP on growth regulation of HPMC, with a focus on cell-cycle machinery such as cyclin D1 and p27^{kip1}, is currently being pursued in our laboratory. We found that the inhibitory effects of DP and cAMP on HPMC may be through the attenuated mitogen-activated protein kinase (MAPK) activity and alterations in expressions of p27^{kip1} and retinoblastoma gene product (pRB) (unpublished data).

The development of PF resulted mainly from an over-

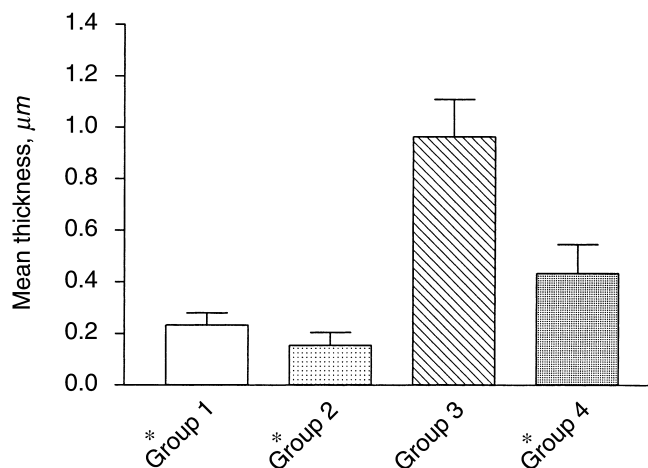


Fig. 8. Comparisons of connective tissue thickness (μm) between different groups. Thickness was assessed using Trichrome-Masson's stain (see **Methods** section). * $P < 0.05$ compared to group 3.

growth of mesothelial cells and/or the accumulation of extracellular matrix. The dual inhibitory effects of DP on HPMC proliferation and collagen synthesis, as demonstrated in our cell culture model, form the rationale for animal experiments to study DP therapy in PF induced subsequently to peritonitis.

In vivo effects

Histological findings of PF in the peritoneum obtained from patients [17] and experimental animals [19] are reportedly characterized by (1) impaired re-mesothelialization of the peritoneum; (2) increased fibro-connective tissue deposition resulting in thickening of the peritoneum and walls of small arteries in the gut mesentery; and (3) the possible prevalence of inflammatory infiltration. In our experiments, the degree of PF in rats treated with a mixed suspension of bacteria plus Cytodex (group 3) was prominent. Both gross development of peritoneal adhesions (Fig. 6) and connective tissue deposits (Figs. 7 and 8) were significantly increased. This group also demonstrated an increase in the mRNA levels of matrix components in peritoneal tissue samples (Fig. 9). In contrast, administration of DP to diseased rats seemed to provide remarkable protection and showed (1) an almost complete suppression of peritoneal adhesions (Fig. 6); (2) less thickness of connective tissue between the mesothelium and abdominal organs (Fig. 7 A–D and 8); (3) attenuation of increased wall thickness of small arteries (Fig. 7 E–H); and (4) a marked decrease in gene expression of collagen I and III in omentum and abdominal wall samples (Fig. 9).

There is no standard method for assessing the degree of peritoneal adhesion. Measurement of connective tissue thickness is the most common method for rating connective tissue deposition (fibrosis) [16]. Nevertheless,

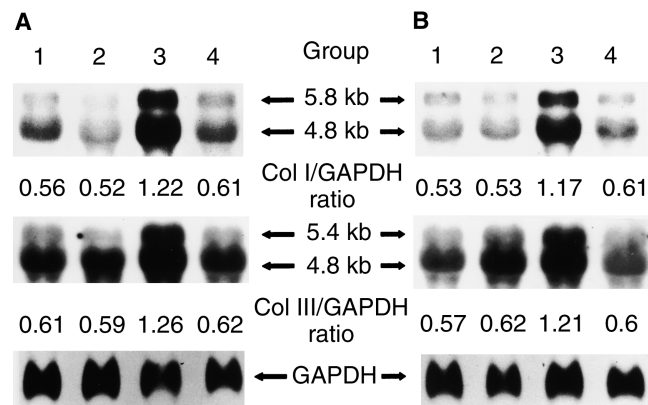


Fig. 9. Expression of collagen $\alpha 1$ (I) and collagen $\alpha 1$ (III) mRNA in adhesion strands of (A) omentum and (B) abdominal wall tissues. Shown are representative Northern blots performed as described in the **Methods** section. Both collagen $\alpha 1$ (I) and $\alpha 1$ (III) mRNA expressions were significantly increased in group 3 (rats treated with bacteria plus Cytodex). In contrast, the expression of both genes was suppressed by DP (group 4).

a gross visual scoring system may provide a quick and semiquantitative assessment of adhesion formation among treatment groups [15]. The present study used gross observation and grading of intraperitoneal changes (Fig. 6) as well as measurement of actual connective tissue thickness (Fig. 8) to show a suppressive effect of DP on adhesion formation. Furthermore, there was a good correlation between the gross observation scores and actual connective tissue thicknesses ($P < 0.01$, Pearson's method, data not shown). Our results suggest that the gross observation scoring system of Mullarniemi et al is convenient and as helpful as the method of actual measurement of connective tissue thickness in evaluating intraperitoneal fibrosis formation [15].

Peritonitis is the main culprit for CAPD patients to develop PF [20]. Experiments have shown that during peritonitis, if the peritoneal fibrinolytic process is not adequate, an ongoing fibrosis process will initiate, resulting in the possible development of adhesions between opposing peritoneal surfaces [21]. The functional fibrinolytic activity is decreased by the severity of peritonitis and existence of foreign bodies [22]. Cytodex has been used as a foreign material to promote local abscess formation in experimental mice [14]. Using this foreign substance, we successfully generated an easily observed and reproducible in vivo rat model of PF. The mechanisms by which Cytodex enhances PF development in our rat model are still not fully understood. Recently, in vitro studies have provided evidence of the importance of mesothelial cells for the balance of peritoneal procoagulant and fibrinolytic activity [23]. The roles of Cytodex and *S. aureus* on alterations in the balance of peritoneal procoagulant and fibrinolytic activity deserve further study.

Our rat model of PF may be somewhat remote from clinical situations in CAPD. However, given that peritonitis due to bacterial infection is one of the leading causes of the development of PF, the results obtained from our animal model may still have clinical implications. Our study represents only a first step for evaluating the effectiveness of this drug on PF in vivo. We did not perform a peritoneal transport assessment to evaluate peritoneal membrane function in the present work because of the following considerations: During peritonitis, peritoneal permeability is increased; however, as we found by gross inspection (Fig. 6), multiple intraperitoneal adhesions and fibrotic retractions may reduce peritoneal volume capacity. Therefore, in the present animal model it would have been difficult to tell whether the changes of peritoneal transport came from effects of peritoneal exchange volume or from peritoneal permeability. A chronic model (that is, one with a longer follow-up period) of the experimental animals and assessment of peritoneal membrane function may help to clarify the relationships between histological and functional changes of the peritoneum.

In conclusion, our study shows, to our knowledge for the first time, that in vitro DP effectively inhibits HPMC proliferation and decreases matrix accumulation. Moreover, it attenuates development of PF in vivo. These results suggest that DP may have potential therapeutic value for the prevention of PF in CAPD patients.

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Reprint requests to Dr. Tun-Jun Tsai, Department of Internal Medicine, National Taiwan University Hospital, No. 7, Chung-Shan South Road, Taipei, Taiwan, Republic of China.
E-mail: paul@ha.mc.ntu.edu.tw

APPENDIX

Abbreviations used in this article are: BHI, brain-heart infusion; BSA, bovine serum albumin; cAMP, cyclic adenosine 3'5'-monophosphate; CAPD, continuous ambulatory peritoneal dialysis; CFU, colony-forming unit; DP, dipyridamole; EIA, enzyme immunoassay; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; GADPH, glyceraldehyde-3-phosphate dehydrogenase; HPMC, human peritoneal mesothelial cells; H-89, N-[2-((p-Bromocinnamyl)amino)ethyl]-5-isoquinoline-sulfonamide; IBMX, 3-isobutyl-1-methylxanthine; IM, intramuscular; IP, intraperitoneal; IVF, continuous intravenous infusion; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PF, peritoneal fibrosis; PKA, protein kinase A; pRB, retinoblastoma gene product.

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